

SOYBEAN SEEDLING ROOT GROWTH PROMOTION BY 1-AMINOCYCLOPROPANE-1-CARBOXYLATE DEAMINASE-PRODUCING PSEUDOMONADS

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ABSTRACT

Pseudomonad producing 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C.4.1.99.4) has been known to promote plant growth by lowering ethylene biosynthesis in higher plants, which can be induced by indole-3-acetic acid (IAA) production. The objective of this study was to examine the ability of IAA-producing *Pseudomonas* isolated from local soil environment (rhizosphere of soybean grown in Plumbon's agricultural area in Cirebon, West Java, Indonesia) to promote soybean root growth in relation to their ACC deaminase activities. The experiments were conducted in growth room and Laboratory of Soil Biology Research, Indonesian Soil Research Institute, Bogor, from January to August 2008. Soybean seeds were inoculated by immersing the seeds for 1 hour in bacterial cell suspension containing approximately 10^8 - 10^9 cells ml^{-1} . The seeds were then germinated for 2 days before planting in growth pouches containing sterilized distilled water. All treated and untreated seeds were grown for 7 days in growth room at 24°C with 1300 lux of light intensity for 12-hour followed by a 12-hour dark period at 22°C. ACC deaminase activity of the isolates was assayed based on their ability to grow in Dworkin-Foster's salt minimal medium containing ammonium sulfate or ACC as a source of nitrogen. Thirteen out of 81 isolates tested significantly increased soybean root length and weight, up to 50% from untreated plants. Of 13 isolates, 11 demonstrated ACC deaminase activities. Two isolates that did not show ACC deaminase activities had lower capacity to produce IAA. The results suggest that the effectiveness of IAA-producing *Pseudomonas* in promoting the growth of the soybean seedlings is associated with their ACC deaminase activities or they produce IAA at low levels.

[**Keywords:** Soybean, root development, ACC deaminase, ethylene, *Pseudomonas*]

INTRODUCTION

The 1-aminocyclopropane-1-carboxylate (ACC) deaminase (E.C.4.1.99.4) is a cytoplasmically localized enzyme produced by some soil bacteria to catalyze the degradation of ACC, a precursor of ethylene, as their source of nitrogen (Jacobson *et al.* 1994; Glick 1995). ACC degradation will ultimately reduce ethylene biosynthesis in the plant. As a senescing

hormone, ethylene stimulates fruit ripening and the aging of flowers; thus, increasing ethylene concentration after seed germination may inhibit seedling growth. Various studies have shown that biosynthesis of ethylene at early stage of plant growth inhibits root development (Glick 1995; Mayak *et al.* 1997; Shah *et al.* 1997) and nodulation of various legumes (Ma *et al.* 2003), and in most cases, weakens plant defense against plant pathogens (Wang *et al.* 2000; Dey *et al.* 2004).

As part of stress hormone that involves in various biotic and abiotic stresses (Glick *et al.* 2007), ethylene biosynthesis in higher plants is induced by indole-3-acetic acid (IAA) production. IAA stimulates the activity of ACC synthase to form ACC, the immediate precursor of plant hormone ethylene in higher plants (Imaseki 1986; Abeles *et al.* 1992; Mayak *et al.* 1997). Previous reports have shown the antagonistic function of ethylene against IAA to prevent overgrowth (gigantism) of plants. The IAA stimulates rooting, but rooting is opposed by ethylene generated by IAA, hence the promotion effects of IAA are offset by the inhibitory effects of ethylene (Lieberman and Kunishi 1972; Mullins 1972; Arshad and Frankenberger 1993). Ample reports on the variable effects of IAA-producing bacteria on various plant growths are well documented. Beyeler *et al.* (1997) found that IAA-overproducing bacteria were deleterious to the growth of wheat and cucumber in autoclaved soil although no such negative effects occurred in non-autoclaved soil. Similar results on hot pepper inoculated by various IAA-producing bacteria showed inconsistent effects on plant growth and yield, both in sterile and non-sterile media, which could be related to ACC deaminase activities (Husen and Saraswati 2005).

The importance of ACC deaminase-producing bacteria for plant growth is to control ethylene biosynthesis in the plants. The dual function of ACC

deaminase for plant growth, i.e. as plant growth promotion and defense against plant pathogens, puts this enzyme as one of the important traits among various beneficial characters of plant growth-promoting bacteria (Cattelan *et al.* 1999; Shaharoon *et al.* 2007). A study by Wang *et al.* (2000) using ACC deaminase-producing *Pseudomonas* and *Enterobacter* proved the ability of these bacteria in enhancing growth and suppressing damping-off of cucumber and root rot diseases of tomato and potato. Saravanakumar and Samiyappan (2007) showed yield improvement of groundnut inoculated by *P. fluorescens* producing ACC deaminase grown in saline soil. The role of many more ACC deaminase-producing bacteria in increasing plant growth is still being studied by using known strains or local isolates obtained from local soil environment.

To date, no studies on ACC deaminase-producing bacteria isolated from the Indonesian soil environment has been reported yet. The challenge is to obtain good isolates that fit with host plants (soybean) but they do not possess any deleterious traits for plant growth. These can be obtained through plant tests as a strategy to screen the best isolates for further tests in their growth promotion activities.

This study examined the ability of IAA-producing *Pseudomonas* isolated from local soil environment to promote soybean seedling root growth under growth room conditions and their ACC deaminase activities *in vitro*. Good isolates obtained can be further developed as plant helpers to increase soybean growth in field soil conditions.

MATERIALS AND METHODS

The study was conducted in growth room and Laboratory of Soil Biology Research, Indonesian Soil Research Institute, Bogor from January to August 2008.

Bacterial Isolates and Growth Media

Eighty two isolates of *Pseudomonas* were used in this study. They were isolated from rhizosphere of soybean grown in Plumbon's agricultural area in Cirebon, West Java, Indonesia. All isolates have been previously screened by Wahyudi *et al.* (2007) for indole-3-acetic acid (IAA) production *in vitro*. Eighty one isolates produced IAA ranging from 0.3 to 23 $\mu\text{g ml}^{-1}$. The isolates were Crb1 to Crb6, Crb8 to Crb37, Crb39 to Crb56, Crb60, Crb74, Crb75, Crb78 to Crb89,

Crb92 to Crb95, Crb102, Crb104, and Crb109 to Crb115. *Pseudomonas* sp Crb38, an isolate that did not produce IAA (Wahyudi *et al.* 2007) was also included in this study as a negative IAA-producer.

All isolates were first grown in King's B medium (KBM) and subsequently transferred to M26 rich medium for bacterial cell production which were used to inoculate plants in root growth assay, or to Dworkin-Foster (DF) salts minimal medium (Dworkin and Foster 1958) supplemented with either ACC (DF-ACC) or ammonium sulfate (DF-Ammonium Sulfate) for ACC deaminase assay. The KBM contained 20 g peptone, 10 ml glycerol, 1.5 g K_2HPO_4 , 1.5 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 15 g agar (for solid media), and 1000 ml distilled water. The M26 broth medium contained 10 g beef extract, 10 g proteose peptone, 5 g NaCl, 15 g agar (for solid media), and 1000 ml distilled water. The composition of DF salts minimal medium were: 4 g KH_2PO_4 , 6 g Na_2HPO_4 , 0.2 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 mg $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 10 $\mu\text{g H}_3\text{BO}_3$, 10 $\mu\text{g MnSO}_4$, 70 $\mu\text{g ZnSO}_4$, 50 $\mu\text{g CuSO}_4$, 10 $\mu\text{g MoO}_3$, 2 g glucose, 2 g gluconic acid, 2 g citric acid, 12 g agar (for solid media), and 1000 ml distilled water. The amount of ACC or ammonium sulfate added to DF salts minimal medium was 0.3033 g or 2 g l^{-1} , respectively.

Except for heat-labile ACC, all media were sterilized by autoclaving for 15 minutes at 121°C and 0.1 Mpa. The heat-labile ACC was filtered-sterilized using 0.2 μm membrane filter (Millipore) before added to the sterilized medium.

Root Growth Promotion Assay

Root growth assay was first conducted as a screening procedure to obtain good isolates that were subsequently assessed for their ACC deaminase activities *in vitro*. The ability of the isolates to increase soybean root growth was assayed by growing the inoculated seedlings in sterile growth pouches under growth room conditions following the protocol described by Liftshitz *et al.* (1987). Since all of the isolates were used in the assay, the experiments were divided into six separate sets of experiments (experiments A to F), each of which consisted of 14-17 tested isolates including the untreated control. Tested isolates used in every set of experiments were selected randomly. Each set of experiments was performed in a completely randomized design with five replications. One replication consisted of three seedlings grown in every growth pouch.

The isolates from KMB were transferred to M26 rich medium to produce high bacterial cells, and then

grown overnight by shaking at 125 rpm. After overnight growth, bacterial cells were centrifuged at 4000 x g for 10 minutes. The cell pellets were washed with 100 mM MgSO₄ and then re-suspended in 100 mM MgSO₄. Prior to being used for seed inoculation, the absorbance of the cell suspension was adjusted to about 0.5 at 780 nm using UV Spectrophotometer which was equal to 10⁸-10⁹ cells ml⁻¹.

Seeds of soybean (*Glycine max* L. Merr.) cv. Wilis were obtained from the Indonesian Center for Agricultural Biotechnology and Genetic Resources Research and Development, Bogor. Soybean seeds, similar in size, were surface disinfected by soaking in 70% alcohol for 1 minute and 1% sodium hypochlorite for 5 minutes, and then rinsed with sterile distilled water several times until no bubbles on the surface of the seeds were visible. The seeds were immersed in either 100 mM MgSO₄ which acted as a blank control (untreated), or suspension of bacterial cells in 100 mM MgSO₄, for 1 hour at room temperature. The treated and untreated seeds were first germinated in sterile Petri dishes containing double wet filter papers for 2 days. Three seedlings with radicle of about 1 cm from the same treatment were planted in each growth pouch, which contained 20 ml of sterilized water. The pouches were placed upright in a rack with two empty pouches at each end of a row. Seedlings were grown for 7 days in growth room at 24°C with 1300 lux of light intensity for 12-hour followed by a 12-hour dark period at 22°C. Root length and root fresh weight of 7-day old seedlings were measured according to Liftshitz *et al.* (1987).

ACC Deaminase Assay

The ACC deaminase activity was assayed for the isolates that significantly increased root growth based on the experiments conducted in growth room conditions. Each isolate was grown on KMB for 24 hours at room temperature, and then transferred into vials containing 10 ml solution of DF, DF-Ammonium Sulfate, and DF-ACC salts minimal medium, and onto solid (agar) plates containing the same medium. The use of DF-Ammonium Sulfate or DF-ACC salts minimal medium was to check whether the ACC deaminase produced by the isolates was part of a constitutive or inducible system in the bacterial metabolism as proposed by Jacobson *et al.* (1994). The use of DF salts minimal medium without supplements as N-free medium was to check whether or not the isolates were able to fix atmospheric dinitrogen.

The inoculated vials were shaken at 125 rpm and bacterial growth was monitored for every 6 hours until 48 hours by measuring optical density at 600 nm (instead of using 570 nm with an ELISA plate reader as conducted by Wang *et al.* 2000). The average values of optical density at 0.05 or more, especially for isolates grown in DF-ACC, indicated that the isolates demonstrated ACC deaminase activities. The inoculated solid media were incubated and observed to confirm the growth of bacterial colonies after 48-hour incubation.

Data were analyzed by the analysis of variance (ANOVA) followed by treatment mean comparison between treated and untreated plants using the least significance difference (LSD; *P* = 0.05). The analyses were conducted using the software of SAS system for Windows v6.12.

RESULTS AND DISCUSSION

Root Growth Promotion

Most isolates used in the study increased soybean root growth. The level of increases was up to 50% from untreated control. However, only 13 out of 81 IAA-producing pseudomonads tested significantly increased root length and/or root fresh weight. They were *Pseudomonas* sp. Crb47 and Crb49 (Fig.1); Crb26, Crb47, and Crb56 (Fig.2); Crb17, Crb31, and Crb86 (Fig. 3), and Crb5, Crb12, Crb24, Crb53, and Crb94 (Fig. 4). None of the isolates used in experiment E significantly increased soybean root growth. Besides these positive effects, most isolates used in experiment F showed lower root development than untreated control indicating that the isolates inhibited soybean root growth (data not shown). *Pseudomonas* sp. Crb38-0 that did not produce IAA and always included in every set of experiments (as a negative control) was also failed to promote soybean root growth.

The variable effects of the isolates from promoting to inhibiting soybean root growth could be related to the IAA concentration in root tissues or the isolates failed to proliferate and grow well in the root zones. The amount of IAA synthesized (by the isolates) from tryptophan or other small molecules present in soybean seed or root exudates (Whipps 1990; Frankenberger and Arshad 1991) may vary based on the different ability of the isolates as described earlier. Previous studies have shown that IAA at low concentrations increased plant growth (Tien *et al.*

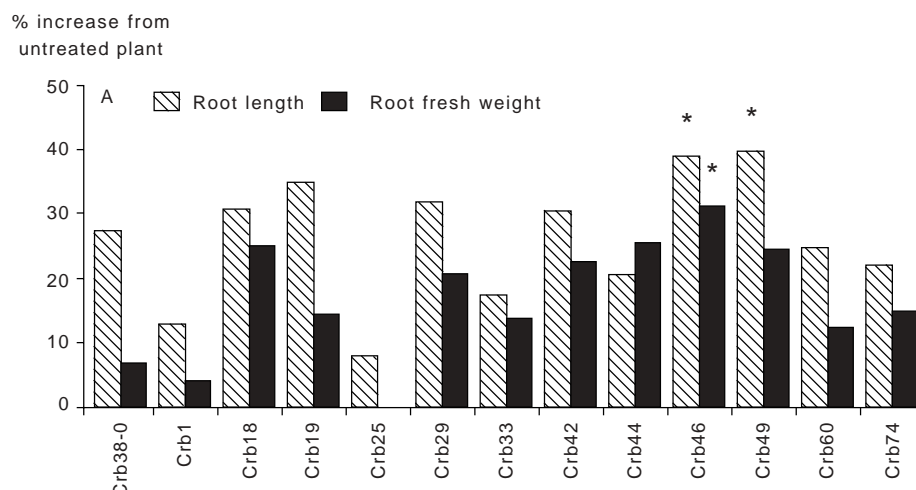


Fig. 1. Root growth of soybean seedlings at 7 days after planting in experiment A. The seeds inoculated with *Pseudomonas* Crb46 and Crb49 significantly increased soybean root length and fresh weight (indicated by * marking in the columns). The values on the y axis represent the percentage of increase from the untreated control, which was considered as 0%.

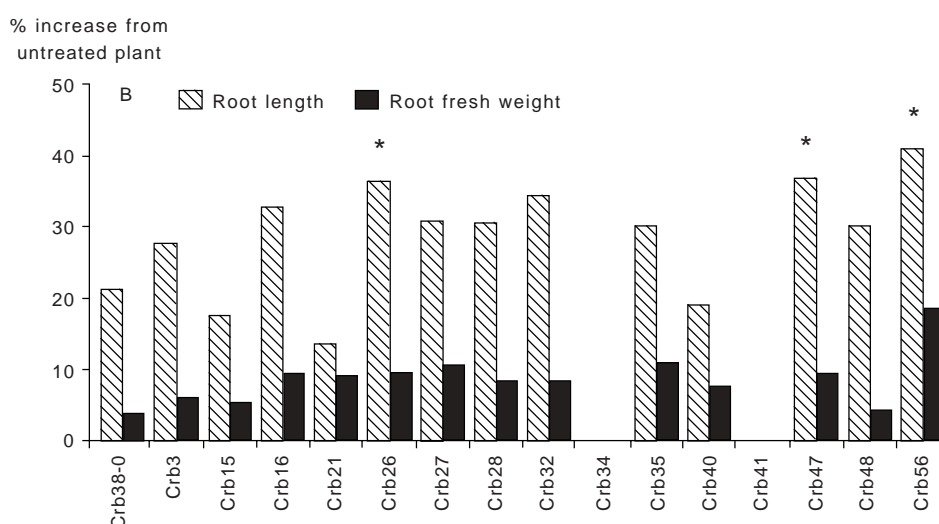


Fig. 2. Root growth of soybean seedlings at 7 days after planting in experiment B. The seeds inoculated with *Pseudomonas* Crb26, Crb47, and Crb56 significantly increased soybean root length and fresh weight (indicated by * marking in the columns). The values on the y axis represent the percentage of increase from the untreated control, which was considered as 0%.

1979; Arshad and Frankenberger 1993) and at high concentrations reduced plant growth (Beyeler *et al.* 1997; Husen and Saraswati 2005). In this study, however, the ability of those 13 best isolates to produce IAA varied from high to low levels (Table 1). This indicates that other mechanism may involve in regulating or optimizing the promoting effects of IAA on root development. In other word, IAA-producing trait per se of a bacterium was not sufficient to conclude its ability to promote plant growth.

ACC Deaminase Activity

Of 13 isolates that increased soybean root development, 11 of them positively produced ACC deaminase based on their ability to grow on DF salts minimal medium supplemented with either ammonium sulfate or ACC as source of nitrogen (Table 1). None of the isolates tested could grow on N-free medium (DF salts minimal medium only) indicating that they were not categorized as diazotrophic bacteria that can fix

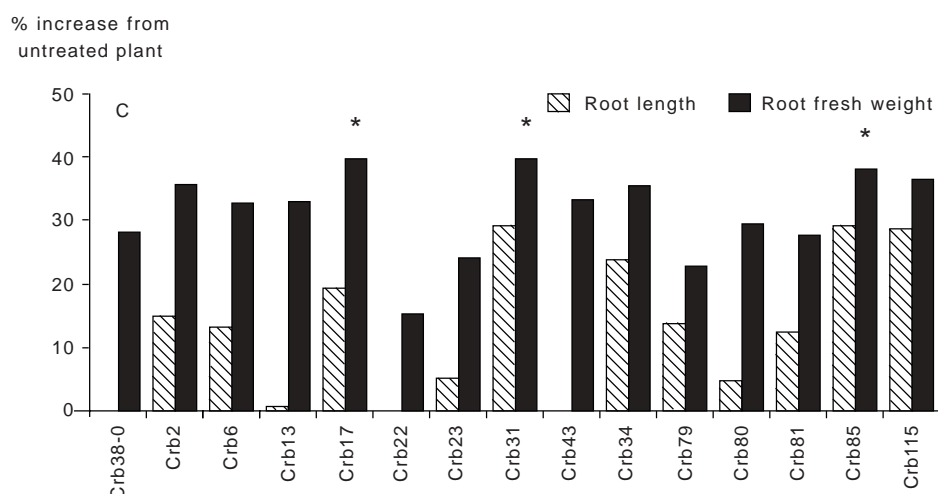


Fig. 3. Root growth of soybean seedlings at 7 days after planting in experiment C. The seeds inoculated with *Pseudomonas* Crb17, Crb31, and Crb86 significantly increased soybean root length and fresh weight (indicated by * marking in the columns). The values on the y axis represent the percentage of increase from the untreated control, which was considered as 0%.

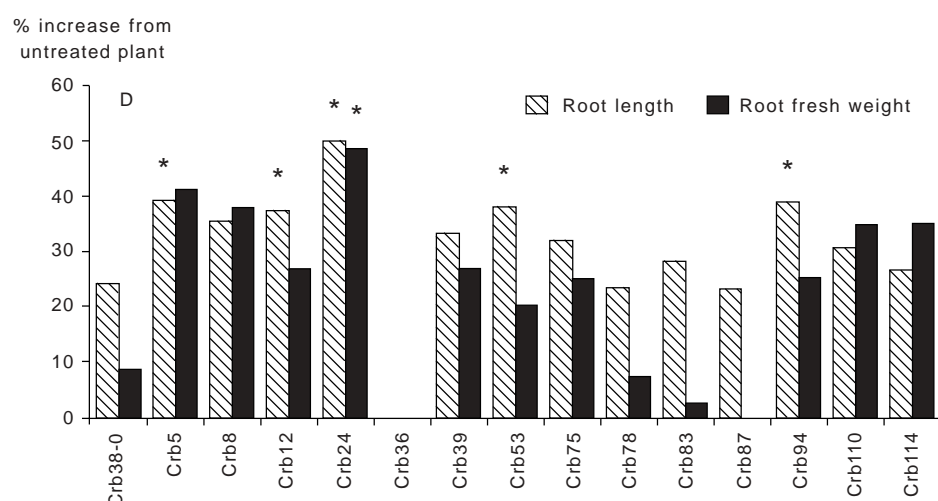


Fig. 4. Root growth of soybean seedlings at 7 days after planting in experiment D. The seeds inoculated with *Pseudomonas* Crb5, Crb12, Crb24, Crb53, and Crb94 significantly increased soybean root length and fresh weight (indicated by * marking in the columns). The values on the y axis represent the percentage of increase from the untreated control, which was considered as 0%.

atmospheric dinitrogen (data not shown). Interestingly, two isolates that did not produce ACC deaminase, i.e. *Pseudomonas* sp. Crb31 and Crb86 had lower capacity to produce IAA in comparison to other isolates suggesting that the levels of IAA produced by these isolates did not inhibit soybean growth. On the other hand, although *Pseudomonas* sp. Crb38-0 produced ACC deaminase, it was failed to promote root growth since it did not have ability to produce IAA. The results demonstrate the physiological role

of ACC deaminase trait in optimizing the promotion effect of IAA-producing bacteria on plant growth.

Cross-talk between ethylene and IAA in relation to plant growth promotion has been described in detail by various researchers. In general, IAA (both endogenous and exogenous produced by plant and bacteria, respectively) stimulates plant cell proliferation and elongation, but it also activates the transcription of ACC synthase that converts S-adenosylmethionine (AdoMet) to ACC and, ultimately, to ethylene by ACC

Table 1. ACC deaminase activity of selected IAA-producing pseudomonads.

<i>Pseudomonas</i> sp.	IAA ($\mu\text{g ml}^{-1}$) ¹	ACC deaminase activity	
		DF + ACC ²	Solid DF + ACC ³
Crb5	12.8	+	+
Crb12	6.7	+	+/-
Crb17	16.0	+	+
Crb24	15.2	+	+
Crb26	12.5	+	+/-
Crb31	5.4	-	-
Crb46	15.8	+	+
Crb47	10.4	+	+
Crb49	16.2	+	+/-
Crb53	16.9	+	+
Crb56	18.5	+	+
Crb86	6.3	-	-
Crb94	1.1	+	+/-
Crb38 (control)	-	+	+/-

¹Previously measured by Wahyudi *et al.* (2007).

²Based on optical density of bacterial growth at 600 nm using UV spectrophotometer in DF salt minimal medium plus ACC as source of nitrogen; (+) the average values of optical density were at 0.05 or more, or (-) at below of 0.05.

³Based on bacterial growth on DF salt minimal medium agar plus ACC as source of nitrogen; (+) bacterial colonies were visible on the plate after 48 hours, (+/-) after 96 hours, or (-) no visible colonies was observed.

oxidase (Kende 1993). Current finding also showed the possibility that ethylene inhibits IAA transport as described by Glick *et al.* (2007). In the presence of ACC deaminase-producing bacteria in the rhizosphere, some of the ACC exuded from plant roots or seeds (to maintain the equilibrium between internal and external plant ACC), is taken up by the bacteria and hydrolyzed to ammonia and α -ketobutyrate (Glick 1995; Glick *et al.* 1998). Thus, bacteria producing ACC deaminase facilitate plant growth by decreasing ethylene production and permitting IAA to stimulate plant cell without the negative effect of increasing ACC.

Taken together, there are two possible opposing trends of ethylene concentration in the plant, i.e. IAA stimulates ethylene synthesis and ACC deaminase lowers ethylene synthesis. Previous study by Mayak *et al.* (1997) proved that the highest levels of ethylene in plant root were observed when either IAA was overproduced or ACC deaminase was not present. In this study, we confirmed that *Pseudomonas* without having ability to produce IAA (Crb38-0) failed to promote soybean seedling root growth and *Pseudomonas* that have capacity to produce IAA as well as ACC deaminase (Crb5, Crb12, Crb17, Crb24, Crb26, Crb46, Crb47, Crb49, Crb53, Crb56, and Crb94) increased

soybean seedling root length and weight; otherwise the levels of IAA produced by the bacteria was low enough to prevent increased concentration of ethylene (Crb31 and Crb86). Further studies are needed to explore the potential use of these bacteria to assist plant in controlling ethylene synthesis in the field which can be induced by various biotic and abiotic (environmental) factors. It includes studies on the prospective use of these bacteria to prevent yield loss of soybean grown in soils with various constraints, such as in peat-soil agriculture (Husen *et al.* 2008).

CONCLUSION

Thirteen out of 81 IAA-producing *Pseudomonas* isolated from the rhizosphere of soybean grown in agricultural area in Plumbon, Cirebon, West Java increased soybean seedling root growth upon inoculation in growth room conditions. Of the 13 isolates, 11 demonstrated ACC deaminase production based on their ability to grow on Dworkin-Foster's salt minimal medium supplemented with either ammonium sulfate or ACC as nitrogen source. Since these IAA-producing bacteria also act as a sink of ACC, applying them to promote soybean growth plays an important role in optimizing the promotion effect of IAA by preventing ethylene biosynthesis in the plant, especially at early stage of plant growth.

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